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Identification of quinone analogues as potential inhibitors of picornavirus 3C protease *in vitro*

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ABSTRACT

Picornaviruses are non-enveloped viruses that represent a large family of positive-sense single-stranded RNA viruses including a number of causative agents of many human and animal diseases such as coxsackievirus B3 (CVB3) and rhinoviruses (HRV). In this study, we performed a high-throughput screening of a compound library composed of ~6000 small molecules in search of potential picornavirus 3C protease ($3C^{pro}$) inhibitors. As results, we identified quinone analogues that effectively inhibited both CVB3 $3C^{pro}$ and HRV $3C^{pro}$ with IC_{50} values in low micromolar range. Together with predicted binding modes of these compounds to the active site of the viral protease, it is implied that structural features of these non-peptidic inhibitors may act as useful scaffold for further anti-picornavirus drug design and development.

Introduction

Human coxsackievirus B3 (CVB3), a member of the family *Picornaviridae*, is considered the most common cause of acute viral myocarditis which can progress to chronic myocarditis and dilated cardiomyopathy leading to heart failure in young adolescents.[1] The picornavirus family includes numerous important pathogens that affect humans and animals such as enteroviruses (EV), human polioviruses (PV), human rhinoviruses (HRV), coxsackieviruses (CV), echoviruses, foot and mouth disease virus and hepatitis A virus.[2–4] Despite their enormous clinical impact and consensus for an urgent need of antivirals, there are no specific therapeutics for picornavirus infections yet.

Picornaviruses are non-enveloped viruses with a positive-sense single-stranded RNA genome.[5] Upon infection, the viral RNA genome is released and translated into a single polyprotein precursor which undergoes successive proteolytic cleavage processes by virus-encoded proteases, mainly $2A^{pro}$ and $3C^{pro}$/$3CD^{pro}$. In particular, the $3C^{pro}$ and its precursor, $3CD^{pro}$, play key roles in virus life cycle as they are required for production of the majority of precursor and mature proteins essential for viral RNA replication and virion assembly.[6,7] Thus, $3C^{pro}$ of picornaviruses has been considered as an attractive target for development of antiviral agents. Several protease inhibitors have been developed by mimicking the peptide substrate of picornavirus $3C^{pro}$. Rupintrivir is a well-known peptidomimetic inhibitor, originally designed to inhibit $3C^{pro}$ of human rhinovirus (HRV) that also shows a broad-spectrum antiviral activity against other picornaviruses.[8–13] However, the application of rupintrivir has been stopped in phase II trial due to limitation to treat the common cold.[14,15]

The aim of this study was to identify selective, low molecular weight, non-peptidic inhibitors of picornavirus $3C^{pro}$. In order to identify novel inhibitors of the CVB3 $3C^{pro}$, we performed a high-throughput screen (HTS) using a library composed of 6000 structurally diverse chemical compounds obtained from Korea Chemical Bank (Daejeon, Republic of Korea) by employing fluorescence resonance energy transfer (FRET)-based *in vitro* protease assay.[16] Briefly, the CVB3 $3C^{pro}$ enzyme was expressed from CVB3-$3C^{pro}$-pET23a plasmid (a gift of Dr. Rolf Hilgenfeld, University of Lübeck, Lübeck, Germany) and purified using immobilized-metal-affinity chromatography (HisTrap FF column, GE Healthcare, UK) as described previously.[17] The workflow of the overall screening procedure is depicted in Supplementary Fig. S1. For the primary screening, each compound was tested in duplicate at a concentration of 40 μM and measured the fluorescence released from

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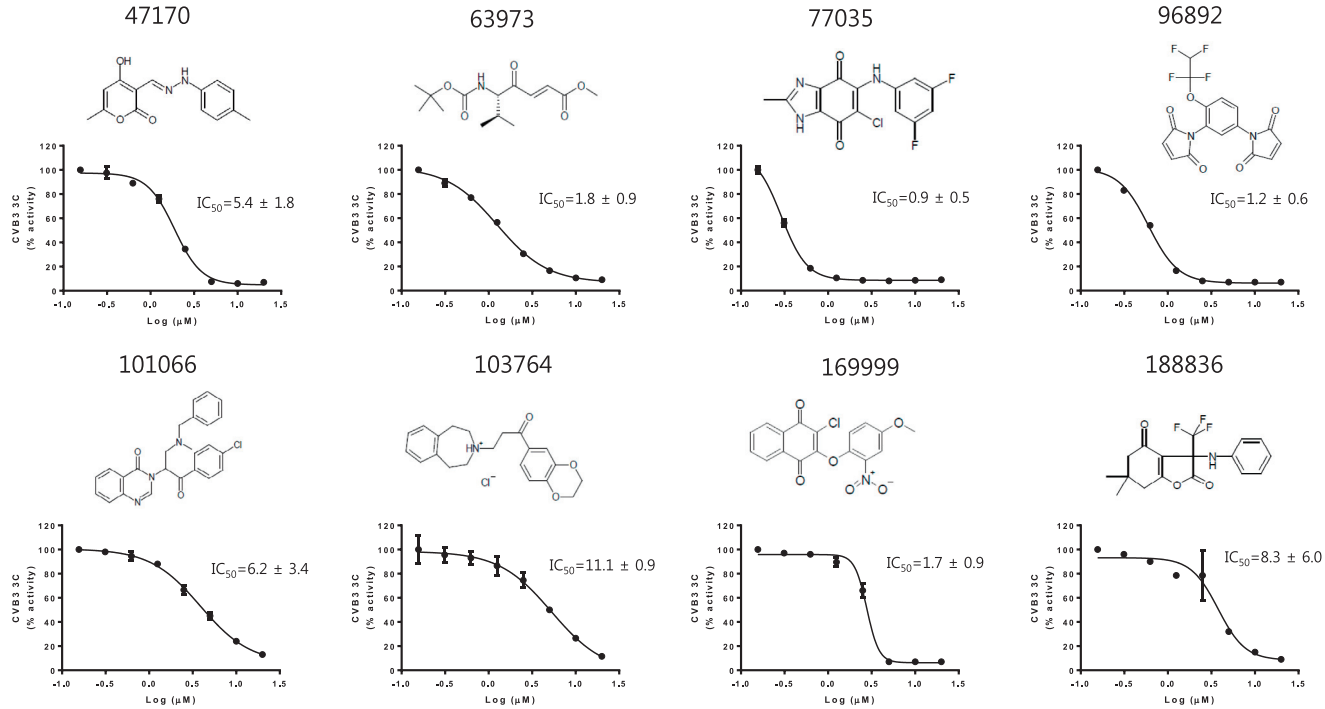

Fig. 1. Chemical structures and dose-response curves (IC_{50}) for the hit compounds against CVB3 $3C^{pro}$. Eight compounds were selected as initial hits from the HTS campaign. The inhibitory activity against CVB3 $3C^{pro}$ was confirmed in a dose-dependent manner using fluorescence resonance energy transfer (FRET)-based *in vitro* protease assay. The chemical structures and compound identification numbers are indicated. The IC_{50} values (mean ± s.d.; μM) are calculated from at least two independent experiments and analyzed by nonlinear regression using GraphPad Prism 6 (GraphPad Software, CA, USA).

the peptide substrate (Dabcyl-KEALFQGPPQFE-Edans, Anygen, Gwangju, Republic of Korea) using Synergy™ H1 Hybrid Multi-Mode microplate reader (BioTek, VT, USA) at excitation and emission wavelengths of 340 nm and 490 nm, respectively. The average Z' score during the primary screen was 0.75 ± 0.06 indicating that the assay was robust and reliable for detection of potential CVB3 $3C^{pro}$ inhibitors (Supplementary Fig. S2A).

From this initial screen, we selected 81 small molecules that showed inhibition of CVB3 $3C^{pro}$ activity greater than 64% (cut-off for anti-protease activity, hit rate 1.35%; Supplementary Fig. S2B). Subsequently, the inhibitory potency of selected compounds were confirmed in an eight-point dose-response protease assay with concentrations ranging from 0.15 to 20 μM. The dose-response experiment identified eight structurally diverse compounds with desirable dose-response activity profiles with inhibitory concentrations (IC_{50}) in the range of 0.9–10.2 μM (Fig. 1). Interestingly, two compounds, 77035 and 169999, with a common quinone scaffold exhibited significant activities against *in vitro* CVB $3C^{pro}$ with IC_{50} values of 0.9 ± 0.5 μM and IC_{50} 1.7 ± 0.9 μM, respectively (Fig. 1).

To firmly determine the relationship between reactivity of quinone with CVB $3C^{pro}$, we selected a series of 77035 and 169999 analogues from another compound library and evaluated for their ability to inhibit the CVB3 $3C^{pro}$ using the above mentioned FRET-peptide substrate enzyme assay. As shown in Table 1, five analogues of 77035 with imidazole quinone scaffold were found to have similar potency against CVB3 $3C^{pro}$ (IC_{50} values < 1 μM) compared to that of 77035. The 77150 and 77125 compounds with a methyl and trifluoromethyl fragment attached to the aniline ring, respectively, showed similar $3C^{pro}$ inhibitory potency compared to that of 77035. Likewise, compounds with a pyridine ring attached to imidazole quinone, such as compounds ID 77072, 77075 and 77076, also displayed excellent anti-3C protease activity with IC_{50} values at nanomolar range. Taken together, the data suggested that the imidazole quinone is the core scaffold responsible for its $3C^{pro}$ inhibitory activity.

In search of 169999 analogues, we interestingly found molecules with quinolone scaffold with improved potency against CVB3 $3C^{pro}$ with IC_{50} values lower to that of 169999 (Table 2). Nevertheless, these results emphasized that quinone as a common structure showed a potent inhibitory activity against CVB3 $3C^{pro}$ suggesting that it likely represents a useful scaffold for designing anti-picornaviral agents.

The structure of $3C^{pro}$ is highly conserved among members of the picornavirus family.[18,19] To evaluate the antiviral activity of the selected compounds against another member of picornavirus family, we examined their anti-$3C^{pro}$ activity using recombinant human rhinovirus (HRV) $3C^{pro}$ (Sino Biological, Shanghai, China) in FRET-peptide substrate assays and SDS-PAGE analysis as described previously.[3,20,21] The FRET-based *in vitro* protease assay revealed that the inhibitory activity of 169999 (IC_{50} 0.85 μM) against HRV $3C^{pro}$ was approximately 10-folds more potent compared to that of 77035 (IC_{50} 8.4 μM) (Fig. 2A and C). Similar results were obtained when the catalytic efficiency of HRV $3C^{pro}$ was shown in the presence of different concentrations of 77035 or 169999 by SDS-PAGE analysis. Specifically, the substrate cleavage was completely suppressed in the presence of 50 μM of 77035 (Fig. 2B) while 5 μM of 169999 (Fig. 2D) was enough to completely inhibit the catalytic activity of HRV $3C^{pro}$. The results from SDS-PAGE analysis indicated that both 77035 and 169999 compounds effectively suppressed not only CVB3 $3C^{pro}$ but also HRV $3C^{pro}$ suggesting broad-spectrum anti-picornavirus activity of the selected compounds.

To further understand the putative binding modes of 77035 and 169999 against CVB3 $3C^{pro}$ and HRV $3C^{pro}$, we performed a flexible docking study using the Schrödinger Suite 2017-1 (Schrödinger, LLC, Portland, OR, USA). The X-ray crystal structures of CVB3 $3C^{pro}$ (PDB: